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Measurements of the proton motive force generated by cytochrome *c* oxidase from *Bacillus subtilis* in proteoliposomes and membrane vesicles

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Cytochrome *c* oxidase from *Bacillus subtilis* was reconstituted in liposomes and its energy-transducing properties were studied. The reconstitution procedure used included Ca^{2+}-induced fusion of pre-formed membranes. The orientation of the enzyme in liposomes is influenced by the phospholipid composition of the membrane. Negatively charged phospholipids are essential for high oxidase activity and respiratory control. Analyses of the proteoliposomes by gel filtration, density gradient centrifugation and electron microscopy indicated a heterogeneity of the proteoliposomes with respect to size and respiratory control. Cytochrome *c* oxidase activity in the proteoliposomes resulted in the generation of a proton motive force, internally negative and alkaline. In the presence of the electron donor, ascorbate/*N,N,N',N'*-tetramethyl-*p*-phenylenediamine/cytochrome *c* or ascorbate/phenazine methosulphate, the reconstituted enzyme generated an electrical potential of 84 mV which was increased by the addition of nigericin to 95 mV and a pH gradient of 32 mV which was increased by the addition of valinomycin to 39 mV. Similar results were obtained with beef-heart cytochrome *c* oxidase reconstituted in liposomes. The maximal proton motive force which could be generated, assuming no endogenous ion leakage, varied over 110 – 140 mV. From this the efficiency of energy transduction by cytochrome *c* oxidase was calculated to be 18 – 23%, indicating that the oxidase is an efficient proton-motive-force-generating system.

The chemiosmotic theory, as proposed by Mitchell [1, 2], states that biological energy converters such as bacteria are able to transform chemical, redox or light energy into the electrochemical energy of protons (Δp). The Δp is composed of an electrical and chemical parameter according to the following relationship: $\Delta p = \Delta\psi - Z\Delta\text{pH}$ (mV), whereby $\Delta\psi$ represents the electrical potential and ΔpH the chemical proton gradient across the membrane (Z equals 2.3 RT/F in which R is the gas constant, T is the absolute temperature and F is the Faraday constant; 2.3 RT/F is 58.8 J/C at room temperature). The electrochemical proton gradient is the driving force for membrane-associated processes such as ATP synthesis and solute transport [3, 4].

Energy conversion in bacteria can be catalyzed by primary proton pumps located in the cytoplasmic membrane. In the gram-positive, aerobically grown bacterium *Bacillus subtilis* the Δp is generated primarily by substrate oxidation via a membrane-bound cytochrome-linked respiratory chain with oxygen as the terminal electron acceptor [5, 6]. The terminal component of the membrane-bound respiratory chain in *Bacillus subtilis* is cytochrome *c* oxidase, which catalyzes electron transfer from cytochrome *c* to dioxygen. This bacterial cytochrome *c* oxidase has been purified to homogeneity [7]. Although *B. subtilis* cytochrome *c* oxidase is structurally less complex than its eukaryotic counterpart, striking spectral and functional similarities were observed between this bacterial and eukaryotic cytochrome *c* oxidases [7 – 9].

The mechanism by which cytochrome *c* oxidase generates a Δp has been one of the central issues in bioenergetics in recent years. Cytochrome *c* oxidase activity can lead to the generation of a Δp by the transfer of electrons from outside to oxygen at the inside and the removal of protons from the intravesicular or liposomal space [10]. In addition to this mechanism of Δp formation, cytochrome *c* oxidase also can couple electron flow to the translocation of protons from the internal to the external phase [11 – 13]. Numerous reports describe how purified cytochrome *c* oxidases of bacterial and eukaryotic origin catalyze vectorial proton translocation [11 – 16]. It has been suggested that even more than one proton per electron can be translocated by cytochrome *c* oxidase [17]. Not all terminal cytochrome *c* oxidases appear to catalyze vectorial proton translocation. For instance no vectorial proton translocation was found with *Rhodopseudomonas sphaeroides* and *Nitrobacter agilis* cytochrome *c* oxidase [18, 19].

Studies on cytochrome *c* oxidases have been concentrated on the molecular mechanism of energy coupling in this enzyme [8 – 20]. Less attention has been paid to the quantification of the electrochemical proton gradient generated by these enzymes. Such quantitative measurements of the protonic potential differences are of ultimate importance for the evaluation of energy transduction by these Δp-generating systems. Several methods have been described to record the trans-

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Abbreviations. Δp, transmembrane electrochemical proton gradient; $\Delta\psi$, transmembrane electrical potential; ΔpH, transmembrane pH gradient; Ph_4P^+, tetraphenyl phosphonium; N-NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl) phosphatidylethanolamine; $Ph(NMe_2)_2$, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; PMS, phenazine methosulphate; PtdEtn, phosphatidyl ethanolamine; PtdGro, phosphatidyl glycerol; Ptd_2Gro, diphosphatidyl glycerol; PtdCho, phosphatidylcholine; S-13, 5 chloro-3-*tert*-butyl-2'-chloro-4'-nitro-salicylanilide; $CF_3OPh_zC(CN)_2$, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; R. C., respiratory control.

Enzyme. Cytochrome *c* oxidase or ferrocytochrome *c*:oxygen oxidoreductase (EC 1.9.3.1).

membranal distribution of indicator molecules (weak acids or bases or lipophilic cations or anions) as probes for the ΔpH or $\Delta\psi$ [21–27]. Also optical probes have been used for measuring the electrical potential or transmembrane pH gradient [28, 42].

In this paper we have quantified both components of the proton motive force generated by cytochrome *c* oxidase from *B. subtilis* in cytoplasmic membranes and in proteoliposomes containing the purified cytochrome *c* oxidase. Several factors which can influence the quantification of these gradients have been unraveled and the properties of the purified cytochrome-*c*-oxidase-containing proteoliposomes have been characterized. The data obtained were compared with Δp measurements in proteoliposomes containing beef-heart cytochrome *c* oxidase.

MATERIALS AND METHODS

Cell growth and preparation of membrane vesicles

Bacillus subtilis W23 was grown at 37 °C with vigorous aeration in a medium containing 0.8% (w/v) trypton (Difco), 0.5% (w/v) NaCl, 25 mM KCl and 150 µl/l micronutrient solution. This micronutrient solution contained: $MnCl_2$ (2.2%), $ZnSO_4 \cdot 7\,H_2O$ (0.05%), H_3BO_3 (0.5%), $CuSO_4 \cdot 5\,H_2O$ (0.016%), $NaMoO_4 \cdot 2\,H_2O$ (0.025%), $Co(NO_3) \cdot 6\,H_2O$ (0.46%) and 0.5 ml/100 ml solution concentrated H_2SO_4. The medium was supplemented with $CaCl_2 \cdot 2\,H_2O$ (50 mg/l) and $FeSO_4 \cdot 7\,H_2O$ (50 mg/l).

Logarithmically growing cells were harvested at an absorbance at 660 nm of 0.8–1.0. Membrane vesicles were prepared as described by Konings et al. [29] and resuspended in a medium containing 50 mM potassium phosphate pH 6.6. If not used immediately the membrane vesicles were stored in liquid nitrogen.

Purification of cytochrome c *oxidases*

Cytochrome *c* oxidase from beef heart was purified as described by Yu et al. [30]. The heme content was 10.4 nmol/mg protein, as determined spectrophotometrically using an absorption coefficient of 13.5 $mM^{-1}\,cm^{-1}$ for reduced minus oxidized heme at the wavelength couple 605/630 nm. Cytochrome *c* oxidase from *B. subtilis* W23 was isolated as described previously by de Vrij et al. [31]. The enzyme preparation had a heme content of 14–15 nmol/mg protein using an absorption coefficient of 16.5 $mM^{-1}\,cm^{-1}$ at the wavelength couple 601/630 nm. Both enzymes were stored in a medium containing 10 mM Hepes/KOH pH 7.0 and 0.7% (w/v) octyl glucoside in liquid nitrogen.

Reconstitution procedures

For the preparation of liposomes different combinations of phospholipids (20 mg/ml) were dispersed in potassium phosphate or Hepes/KOH of pH 7.0 and varying ionic strengths which were supplemented with 0.7% (w/v) octyl glucoside. These suspensions were sonicated to clarity under a constant stream of nitrogen gas at 4 °C using a probe-type sonicator (MSE).

Liposomes were dialyzed at 4 °C for 4 h against a 1000-fold volume of the buffer in which they were prepared. Dialysis buffer was changed twice. Liposomes prepared in Hepes/KOH were, after the first dialysis step, dialysed for 4 h against this buffer supplemented with 5 mM $CaCl_2$. In the presence of calcium, fusion of the preformed liposomes is induced [32]. Subsequently dialysis against a 1000-fold volume of Hepes/KOH without $CaCl_2$ was performed for 14 h at 4 °C in order to remove excess calcium. The diameter of the liposomes obtained with this procedure varied between 50 nm and 300 nm as determined in electron micrographs of negatively stained liposomes. The diameter of liposomes dialysed without Ca^{2+} was between 40 nm and 60 nm.

The proteoliposomes containing cytochrome *c* oxidase were prepared by a modification of the procedure of Hinkle et al. [33]. Beef-heart cytochrome *c* oxidase was incorporated in the liposomes using 0.22 nmol heme *a*/mg phospholipid and for *B. subtilis* cytochrome *c* oxidase 0.4 nmol heme *a*/mg phospholipid, unless stated otherwise.

Incorporation of both enzymes was performed in Hepes/KOH pH 7.0 supplemented with $CaCl_2$ during the dialysis as described above.

Electrical potential and the transmembrane pH gradient

The $\Delta\psi$ (interior negative) was determined from the distribution of tetraphenyl phosphonium (Ph_4P^+) across the membranes using a Ph_4P^+-sensitive electrode [25]. A correction for concentration-dependent probe binding was applied according to the model described by Lolkema et al. [27]. For the $\Delta\psi$ measurements the oxygen-saturated reaction mixtures contained 10 mM Hepes/KOH pH 7.0, 45 mm KCl, 2 µM Ph_4P^+ and varying amounts of proteoliposomes. All measurements were performed at 20 °C.

Valinomycin-mediated potassium diffusion potentials were imposed across the liposomal membrane by diluting liposomes containing different concentrations of potassium and 5 nmol valinomycin/mg phospholipid with a medium of the same ionic strength, in which sodium ions substituted the potassium ions.

To determine the (a)symmetry of Ph_4P^+ binding to the artificial membranes, liposomes were used which were prepared from PtdCho/Ptd_2Gro (molar ratio 1:1) in a medium containing 50 mM sodium phosphate pH 7.0 or in a medium containing 10 mM Hepes/NaOH pH 7.0, 45 mM NaCl and with 5 mM $CaCl_2$ included in the second dialysis step. Binding to the external surface was determined after 100-fold dilution of the liposomes in the presence of valinomycin (5 nmol/mg phospholipid) with the medium in which they were prepared and in which sodium ions were replaced by potassium ions. Under these conditions an artificial membrane potential, inside positive, will be generated. The assumption is made here that under these conditions binding of Ph_4P^+ occurs solely to the external surface of the membrane. Binding of Ph_4P^+ to both external and internal surfaces of the liposomal membranes was determined after 100-fold dilution of the liposomes in the presence of valinomycin with the same buffer in which the liposomes were prepared.

The transmembrane proton gradient (interior alkaline) was determined from the distribution of [^{14}C]acetate using automated flow dialysis as described [24]. The internal volume of the liposomes obtained by calcium-induced fusion was determined with the method described by Kendall et al. [34], from the entrapped content of calcein, and was calculated to be 5 µl/mg phospholipid. Internal volumes of the other liposome preparations were assumed to be 1.5 µl/mg phospholipid, whereas the internal volume of *B. subtilis* membrane vesicles was 3.0 µl/mg membrane protein [35].

Internal pH changes were measured by following the fluorescence of entrapped pyranine [28]. To incorporate pyranine

into proteoliposomes and membrane vesicles 100 nmol pyranine was added to 0.5 ml proteoliposomes (20 mg phospholipid/ml) or 0.5 ml membrane vesicles (20 mg membrane protein/ml) and rapidly mixed. These suspensions were rapidly frozen in liquid nitrogen and subsequently slowly thawed at room temperature. To remove external pyranine the proteoliposomes or membrane vesicles were chromatographed over a Sephadex G-50 column (5 cm × 1-cm diameter) pre-equilibrated with 10 mM Hepes/KOH pH 7.0 containing 45 mM KCl and eluted with the same buffer. Fluorescence changes were measured using the excitation/emission couple 460/508 nm with a Perkin-Elmer fluorimeter. The system was calibrated by titrating acid or base in the uncoupled proteoliposomes or membrane vesicles. Proteoliposomes were uncoupled by the addition of 10 nM 5-chloro-3-*tert*-butyl-2′-chloro-4′-nitrosalicylanilide (S-13), whereas for uncoupling of the membrane vesicles from *B. subtilis* 0.5 μM S-13 was used.

CHARACTERIZATION OF PROTEOLIPOSOMES

Density gradient/gel filtration

Proteoliposomes were used which were composed of PtdCho and Ptd_2Gro in a molar ratio of 1:1 in which 0.4 nmol heme *a* of *B. subtilis* cytochrome *c* oxidase/mg phospholipid was incorporated. As a phospholipid marker, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine (N-NBD-PE) (0.5 mol % of total phospholipid) was incorporated in the proteoliposomes. Discontinuous Ficoll-400 gradients were formed in a vertical rotor of Sorvall (SS-90). The gradients were formed in 10 mM Hepes/KOH of pH 7.0 containing 45 mM KCl from the following Ficoll concentrations (% w/v): 30% (2 ml), 15% (3.5 ml), 7.5% (3.5 ml), 5% (4.5 ml), 4% (4.5 ml), 3% (4.5 ml), 2% (4.5 ml).

Proteoliposomes (0.6 ml), containing 4 mg phospholipid in 1% Ficoll were layered on top of the gradient. The centrifugation was performed for 4 h at 48000 × *g* at 4 °C. After centrifugation fractions of 2.5 ml were analyzed for cytochrome *c* oxidase activity, respiration control index and refractive index. N-NBD-PE fluorescence of the fractions was measured using excitation and emission wavelengths of 475 nm and 530 nm, respectively [36].

Gel filtration of the proteoliposomes was performed using a Bio-Gel A 150 M (Bio-Rad) column (30 × 2 cm) which was equilibrated with 10 mM Hepes/KOH pH 7.0, containing 45 mM KCl. Proteoliposomes (2 ml) were loaded on this column and 0.8-ml fractions were analyzed for cytochrome *c* oxidase activity, respiration control index and phospholipid content as described above. To determine the size of the proteoliposomes fractions were pooled and analyzed by electron microscopy of negatively stained specimens.

Analytical procedures

Cytochrome *c* oxidase activity was measured spectrophotometrically at room temperature by following the decrease in absorbance of the α peak of cytochrome *c* using an absorption coefficient (reduced minus oxidized) of 19.5 mM^{-1} cm^{-1} (550 − 540 nm) [37].

The orientation of cytochrome *c* oxidase in the proteoliposomes was measured as described by Casey et al. [38]. Spectrophotometric determinations were performed with an Aminco-DW-2a spectrophotometer.

The respiratory control (R. C.) index was determined from the ratio of cytochrome *c* oxidase activity measured under saturating substrate conditions (i.e. 20 μM cytochrome *c*) in the presence and absence of 2.0 μM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone $[CF_3OPh_2C(CN)_2]$ and 0.4 μM valinomycin. Protein was determined according to Lowry et al. [39] using bovine serum albumin as a standard.

Oxygen consumption by membrane vesicles of *B. subtilis* was measured polarographically in a 2.0-ml reaction vessel using a Clark-type electrode (Yellow Springs Instruments, Co.). The oxygen-saturated reaction mixture contained 50 mM potassium phosphate and 5 mM $MgSO_4$. As electron donors, sodium ascorbate (10 mM) in combination with $Ph(NMe_2)_2$ (200 μM) and yeast cytochrome *c* (10 μM) were used.

Materials

Octyl glucoside, yeast cytochrome *c* (type VIII) and crude asolectin were purchased from Sigma Chemical Co. Asolectin was prepared from crude asolectin as described [40]. N-NBD-PE was kindly provided by Dr J. Wilschut (Department of Physiological Chemistry, University of Groningen, The Netherlands).

RESULTS

The electrical potential in liposomes

For the measurements of the electrical potential we used electrodes selectively sensitive for the lipophilic cation tetraphenyl phosphonium. Lipophilic cations, however, bind to biological membranes. Therefore, to quantify the electrical potential across the biological membranes we used a correction model in which this probe binding is taken into account [27]. The electrical potential is calculated from the adapted equation:

$$\Delta\psi = Z\log\left[\frac{c_0(c_e-1)^{-1} + x(1-0.5f_{cm}K_{cm}}{x(1+0.5f_{cm}K_{cm})}\right]$$

where c_0 represents the probe concentration before binding or uptake has occurred, c_e represents the external probe concentration after the generation of an electrical potential, x represents the fractional internal volume and the term f_{cm} K_{cm} is the binding constant of the probe.

The binding constants were determined of liposomes composed of different phospholipids and with different sizes in media of different ionic strengths (Table 1). Binding of Ph_4P^+ appeared to be strongly dependent on the phospholipid composition and occurred especially to negatively charged phospholipids such as Ptd_2Gro. At increasing ionic strength the apparent Ph_4P^+ binding decreased. In Table 1 the results of different liposomal preparations are shown. Liposomes prepared by the addition of 5 mM $CaCl_2$ to one of the dialysis buffers bind a significant amount of the calcium (35 mmol Ca^{2+}/mol phospholipid). A significantly lower binding of Ph_4P^+ to these larger Ca^{2+}-containing liposomes was observed. Relatively low concentrations of bivalent cations (5 mM $MgSO_4$) also drastically decreased the binding of Ph_4P^+ to the liposomes.

In order to determine whether the electrical potential calculated with this correction model gives a realistic value, a valinomycin-induced potassium diffusion potential was imposed in two liposomal preparations (PtdCho/Ptd_2Gro molar

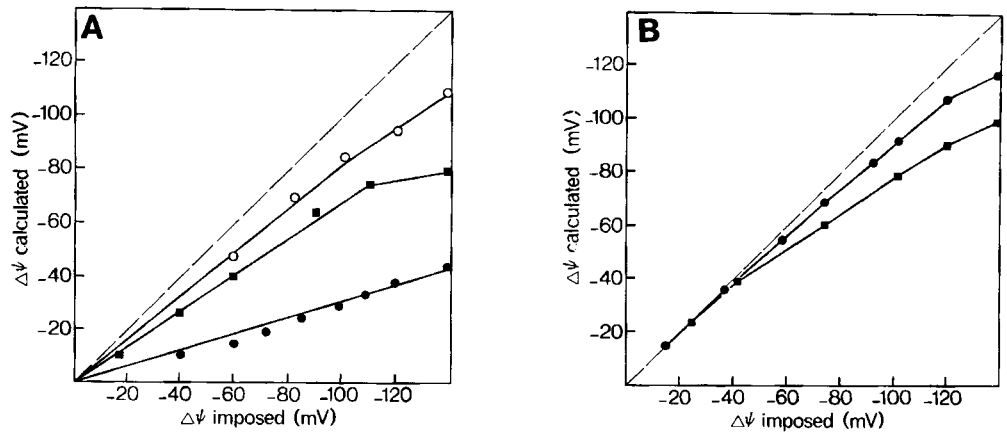

Fig. 1. *Relation between imposed and calculated $\Delta\psi$ in various liposomal preparations.* Valinomycin-mediated potassium diffusion potentials were applied as described in Methods. The $\Delta\psi$ was calculated using the correction for probe binding as described in the text. (A) Liposomes (PtdCho/Ptd_2Gro molar ratio 1:1) prepared with exclusion of $CaCl_2$ during dialysis at different ionic strength: (●) 15 mM potassium phosphate, pH 7.0; (■) 75 mM potassium phosphate, pH 7.0; (○) 400 mM potassium phosphate, pH 7.0. (B) Liposomes (PtdCho/Ptd_2Gro molar ratio 1:1) prepared with inclusion of $CaCl_2$ during dialysis: (■) 10 mM Hepes/KOH, pH 7.0, containing 45 mM KCl; (●) 10 mM Hepes/KOH, pH 7.0, containing 200 mM KCl

Table 1. *Binding constants in different liposome preparations*
Binding constants for Ph_4P^+ ($f_{cm}K_{cm}$; see equation in text) were determined by the addition of 0.1 ml liposomes (20 mg phospholipid/ml) to 1 ml buffer containing 4 μM Ph_4P^+. Binding was determined after a correction was applied for dilution of the liposomes. Some liposomes were prepared with inclusion of $CaCl_2$ in the dialysis buffer. KP_i = potassium phosphate

Membrane composition (molar ratio)	$CaCl_2$	Medium composition	Binding constant
Ptd_2Gro	–	75 mM KP_i pH 7.0	3990
PtdCho/Ptd_2Gro (1:1)	–	15 mM KP_i	6940
PtdCho/Ptd_2Gro (1:1)	–	75 mM KP_i	1735
PtdCho/Ptd_2Gro (1:1)	–	400 mM KP_i	365
PtdCho/Ptd_2Gro (1:1)	+	10 mM Hepes/K^+, 50 mM KCl pH 7.0	115
PtdCho/Ptd_2Gro (1:1)	+	10 mM Hepes/K^+, 200 mM KCl	71
Asolectin	+	10 mM Hepes/K^+, 50 mM KCl	56
Asolectin	+	10 mM Hepes/K^+, 50 mM KCl, 5 mM $MgSO_4$	14

ratio 1:1) with different average diameters (Fig. 1 A, B). In both preparations the calculated $\Delta\psi$ reached higher values when the ionic strength was increased. In liposomal preparations with a diameter of 40–60 nm a discrepancy of imposed and calculated $\Delta\psi$ was found even at low applied potentials (Fig. 1 A). In the Ca^{2+}-liposomes at high ionic strength a very good correlation was observed between the imposed and calculated $\Delta\psi$ at low diffusion potentials (Fig. 1 B). The correlation decreased somewhat at high diffusion potentials, possibly due to saturation of probe binding. These data clearly indicate that with a decrease of Ph_4P^+ binding an increase of the correlation between imposed and calculated potentials is observed.

Another factor which could influence the calculation of $\Delta\psi$ in these liposomes is the (a)symmetry of Ph_4P^+ binding. The correction model is based on the assumption that binding to external and internal surfaces are equal. A comparison of binding in the presence or absence of an imposed reversed $\Delta\psi$ indicated that 75–80% of the binding to small liposomes occurred at the external surface area of the membrane. In contrast in large Ca^{2+}-liposomes only 55–66% of the total binding occurred at the external surface.

In situations where Ph_4P^+ binding was extremely low (asolectin liposomes in 10 mM Hepes/KOH containing 45 mM KCl and 5 mM $MgSO_4$ at pH 7.0) the $\Delta\psi$ calculated corresponded to the imposed potential of −120 mV within 10 mV at Ph_4P^+ concentrations upto 5 μM. To circumvent problems in quantifying the $\Delta\psi$ across liposomal membranes in which a Δp-generating system was incorporated, we used proteoliposomes made from asolectine and with $CaCl_2$ included in the dialysis buffer. Measurements were performed in media with relatively high ionic strength. The binding constants observed with *B. subtilis* membrane vesicles were relatively low (f_{cm} K_{cm} 20–40). Therefore no specific care had to be taken here to circumvent high binding of Ph_4P^+ to the membranes.

Electrical potential in cytochrome-c-oxidase-containing liposomes

Oxidation of externally added reduced cytochrome *c* by cytochrome *c* oxidase incorporated in liposomes generates a proton motive force, inside alkaline and negative [41]. Successful incorporation of cytochrome *c* oxidase in the liposomal membrane can be measured conveniently from the degree of acceleration of enzyme turnover after collapse of the Δp with ionophores. The highest respiratory control indices (R. C. index = 3) were obtained in membranes containing negatively charged phospholipids (Ptd_2Gro and PtdGro). Cytochrome *c* oxidase activity was also highest in liposomes containing negatively charged phospholipids (data not shown). Optimal incorporation of *B. subtilis* cytochrome *c*

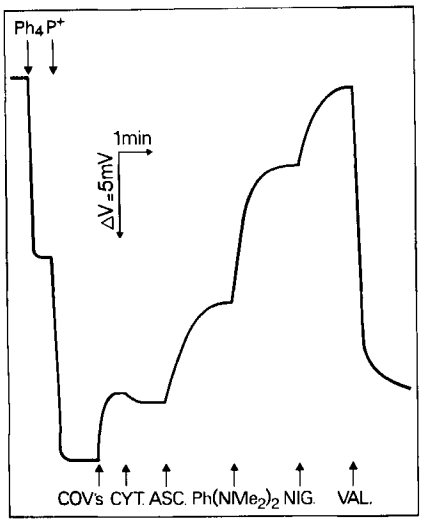

Fig. 2. *Tetraphenylphosphonium (Ph_4P^+) uptake by proteoliposomes containing* B. subtilis *cytochrome* c *oxidase.* The proteoliposomes (COV's) (1.42 mg asolectine) were added to 1 ml oxygen-saturated medium containing 2 μM Ph_4P^+. Ascorbate (ASC., 2 mM), $Ph(NMe_2)_2$ (200 μM) and cytochrome *c* (CYT., 10 μM), followed by nigericin (NIG., 10 nM) and valinomycin (VAL., 0.5 μM) were added as indicated. Calibration of the electrode was performed twice by twofold increase of the Ph_4P^+ concentration

Table 2. *Quantification of Δψ (interior negative) generated in liposomes containing beef-heart or* B. subtilis *cytochrome* c *oxidase*
Proteoliposomes were prepared as described in Methods. *B. subtilis* cytochrome-*c*-oxidase-containing liposomes (1.42 mg asolectin) and beef-heart cytochrome-*c*-oxidase-containing liposomes (0.4 mg asolectin) were used in a total volume of 1 ml. Ionophores: nig. = nigericin (10 nM), val. = valinomycin (0.5 μM)

Electron donor	Ionophore	Δψ in proteoliposomes of	
		B. subtilis	beef heart
		mV	
Ascorbate (2 mM) + cytochrome *c* (10 μM)	–	−56	−83
Ascorbate (2 mM) + cytochrome *c* (10 μM) + (200 μM) $Ph(MNe_2)_2$	–	−84	−92
	+ nig.	−95	−101
	+ val.	0	0
Ascorbate (10 mM) + PMS (20 μM)	–	−86	−73
	+ nig.	−96	−81
	+ val.	0	0

oxidase (75% cytochrome *c* binding sites located at the external surface) was achieved in membranes with a phospholipid composition comparable to the composition of the cytoplasmic membrane (i.e. PtdEtn/PtdGro/Ptd_2Gro in a molar ratio 2:5.5:2.5).

Proteoliposomes containing *B. subtilis* cytochrome *c* oxidase accumulated Ph_4P^+ upon addition of a suitable electron donor (Fig. 2). The most commonly used electron donor is reduced cytochrome *c*. The highest turnover rates of *B. subtilis* cytochrome *c* oxidase were obtained with yeast cytochrome *c* [31]. With ascorbate (2 mM) and cytochrome *c* (10 μM) a significant *Δψ* was generated (Fig. 2). The *Δψ* increased drastically upon addition of $Ph(NMe_2)_2$ (200 μM). $Ph(NMe_2)_2$ accelerates electron transfer from ascorbate to cytochrome *c*, which appears to be the rate-limiting step in the electron transfer reaction. The same observation was made with beef-heart cytochrome *c* oxidase proteoliposomes (Table 2). Electron flow to cytochrome *c* was also increased by increasing the ascorbate concentration to 30 mM. Under these conditions the *Δψ* generated was comparable to the *Δψ* generated with ascorbate/$Ph(NMe_2)_2$/cytochrome *c* (Table 2).

Reduced phenazine methosulphate (PMS) could also function as electron donor. The addition of ascorbate (10 mM) in combination with PMS (20 μM) to *B. subtilis* cytochrome *c* oxidase proteoliposomes resulted in a rapid *Δψ* generation to a comparable extent as with the electron donor system ascorbate/$Ph(NMe_2)_2$/cytochrome *c* (Table 2). In beef-heart cytochrome *c* oxidase proteoliposomes, PMS seems to be a somewhat less effective electron donor than reduced cytochrome *c* (Table 2).

The ionophore nigericin catalyzes electrical neutral exchange of protons for potassium, thereby collapsing the transmembrane pH gradient. In systems in which an active proton pump functions, this decrease of *Δp* is often compensated by an increase of the *Δψ*. This was also observed in proteoliposomes upon addition of 10 nM nigericin (Table 2). Higher concentrations of nigericin had uncoupling effects and led to a decrease of the *Δψ*. An increased *Δψ* was also observed when the *Δ*pH was collapsed by acetate (30 mM), a membrane-permeable weak acid or by triphenyltin (30 nM), a chloride/hydroxyl ion-exchanger (data not shown). Valinomycin (0.5 μM), a K^+ ionophore, completely dissipated the *Δψ*.

The maximum proton motive force which can be generated in these proteoliposomes will be determined by the pump activity and the endogenous ion-leakage processes. To obtain the maximal *Δψ* (*Δp*) low concentrations of nigericin were added to the proteoliposomes. Different turnover rates of the cytochrome *c* oxidase were obtained by varying the concentration of cytochrome *c*. The reduction state of cytochrome *c* was kept constant (50% reduced) under all conditions by varying the amount of added ascorbate. In this way the free energy (*ΔG*) of the reaction was maintained constant. The H^+ leakage of the membrane was increased by the addition of increasing amounts of the uncoupler S-13. The assumption was made that the proton permeability of the membrane increased linearly with the amount of added uncoupler. The results are shown in Fig. 3. The reciprocal of the generated *Δψ* was plotted against the uncoupler concentration. The intercept of all lines represents a situation in which no endogenous proton permeability of the membrane exists. The extrapolated maximal *Δψ* (*Δp*), varied between −110 mV and −140 mV and the endogenous proton leakage of the membranes was comparable to a leakage created in these membranes by the addition of 0.125 nM S-13. For beef-heart proteoliposomes a comparable value of maximum *Δp* was found (data not shown).

The measurements of the *Δψ* in these proteoliposomes with Ph_4P^+ as a probe molecule could be disturbed by secondary effects of Ph_4P^+ on, for example, membrane permeability

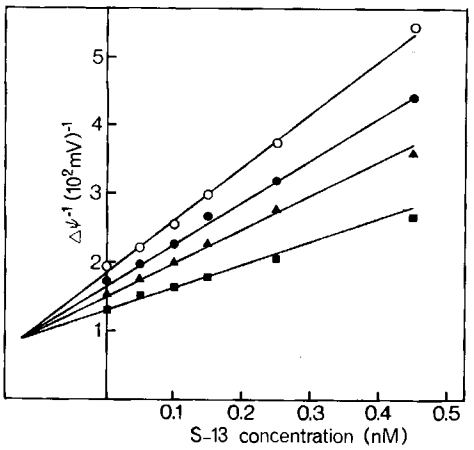

Fig. 3. *Relation between reciprocal of $\Delta\psi$ (Δp) and uncoupler concentration (S-13) in proteoliposomes containing* B. subtilis *cytochrome* c *oxidase.* The $\Delta\psi$ generated by the proteoliposomes, in the presence of nigericin (10 nM), was determined as described in Methods using various concentrations of ascorbate and cytochrome *c* as electron donor. The $\Delta\psi$ was titrated with increasing concentrations of uncoupler S-13. (○) Ascorbate (2 mM), cytochrome *c* (2 μM); (●) ascorbate (3 mM), cytochrome *c* (6 μM); (▲) ascorbate (4.5 mM), cytochrome *c* (10 μM); (■) ascorbate (7 mM), cytochrome *c* (20 μM)

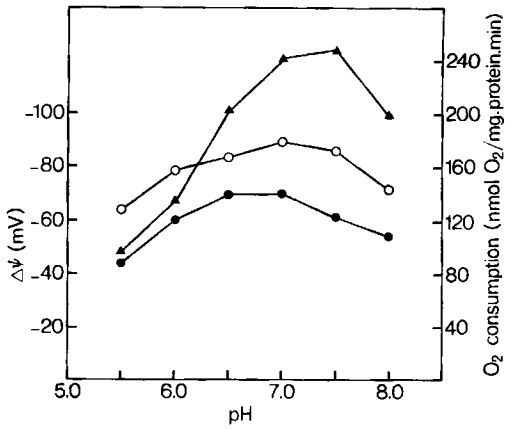

Fig. 4. *pH dependence of cytochrome* c *oxidase activity and generation of $\Delta\psi$ by cytochrome* c *oxidase in* B. subtilis *cytoplasmic membranes.* Oxygen consumption and Ph_4P^+ uptake were measured simultaneously in a 2-ml closed vessel containing *B. subtilis* membrane vesicles (1.8 mg protein) in oxygen-saturated 50 mM potassium phosphate and 5 mM $MgSO_4$ at various pH values. Ascorbate (10 mM), $Ph(NMe_2)_2$ (200 μM) and cytochrome *c* (10 μM) were added as electron donor. Nigericin (0.5 μM) was used to dissipate the pH gradient. (▲) Oxygen consumption rate; (●) $\Delta\psi$ in the absence of ionophores; (○) $\Delta\psi$ in the presence of nigericin (0.5 μM)

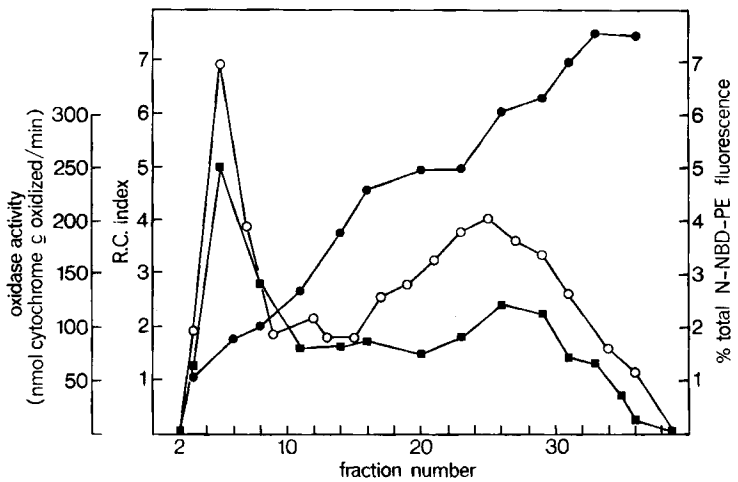

Fig. 5. *Gel filtration (Bio-Gel A 150 M) of proteoliposomes containing* B. subtilis *cytochrome* c *oxidase.* Proteoliposomes (2 ml, PtdCho/Ptd_2Gro molar ratio 1:1) were loaded on the gel filtration column as described in Methods and fractions were analyzed. Liposomal phospholipid was determined from the fluorescence of N-NBD-PE which was incorporated into the proteoliposomes as described. (●) R. C. index; (○) N-NBD-PE fluorescence; (■) cytochrome *c* oxidase activity measured in the presence of $CF_3OPh_2C(CN)_2$ (2 μM) and valinomycin (0.4 μM)

(uncoupling phenomena) or direct effects of Ph_4P^+ on cytochrome *c* oxidase activity. Up to 5 μM Ph_4P^+ no significant decrease of the calculated $\Delta\psi$ was observed but higher Ph_4P^+ concentrations tended to decrease the $\Delta\psi$ and led also to a decrease of the R. C. index. The membrane-associated cytochrome *c* oxidase activity, measured in the uncoupled state, was not effected by Ph_4P^+ up to 5 mM.

Electrical potential in membrane vesicles

The $\Delta\psi$ generated by *B. subtilis* cytochrome *c* oxidase in its natural environment, the cytoplasmic membrane, was also recorded (Fig. 4). Cytochrome *c* oxidase activity was measured polarographically in the presence of ascorbate/$Ph(NMe_2)_2$/cytochrome *c*. The activities were optimal in the pH range 7–7.5. Even without the addition of cytochrome *c*, the combination of ascorbate/$Ph(NMe_2)_2$ resulted in high rates of oxygen consumption, due to the presence of endogenous cytochrome *c* in the cytoplasmic membranes. The $\Delta\psi$ in the absence of nigericin followed the oxidase activity. In the presence of nigericin (0.5 μM), the $\Delta\psi$, which is the only component of the Δp, varied significantly less than the turnover rate of the enzyme in the pH range studied. Under the conditions employed no R. C. index was observed in the cytoplasmic membranes, despite the generation of high Δp values.

Characterization of the proteoliposomes

For the quantification of the electrical potential it is important to know whether all liposomes participated in the genera-

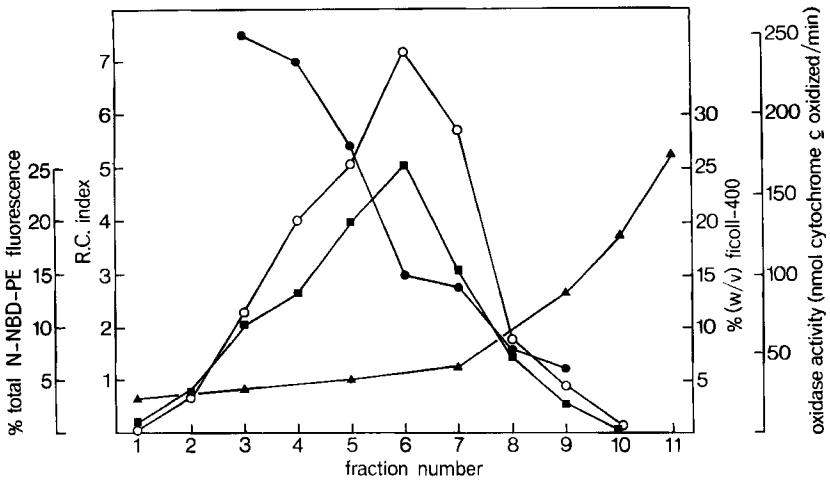

Fig. 6. *Ficoll density gradient centrifugation of proteoliposomes containing* B. subtilis *cytochrome* c *oxidase.* Ficoll density gradient centrifugation was performed as described in Methods using 0.6 ml proteoliposomes (PtdCho/Ptd_2Gro molar ratio 1:1). After fractionation, fractions were analyzed for R.C. index (●), cytochrome *c* oxidase activity (■), liposomal phospholipid as indicated by N-NBD-PE fluorescence (○) and refractive index (▲)

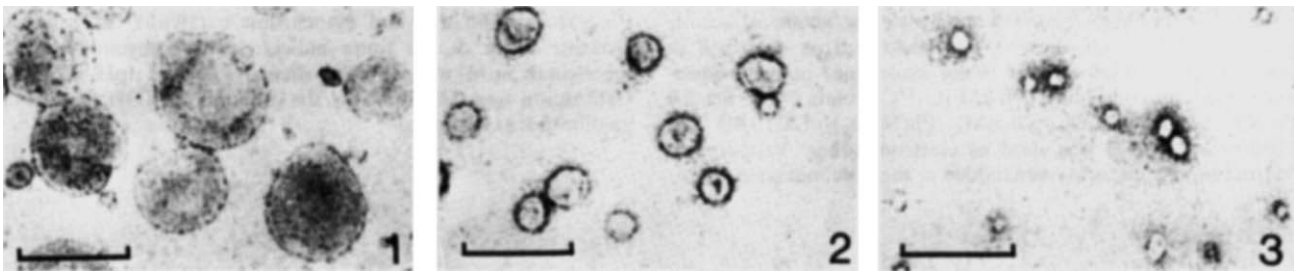

Fig. 7. *Electron micrographs of negatively stained preparations obtained from the pooled fractions from the gel filtration column.* (1) Fractions 1–5; (2) fractions 11–15; (3) fractions 30–35. Bar represents 0.2 μm

tion of a *Δp*. Ca^{2+}-proteoliposomes containing *B. subtilis* cytochrome *c* oxidase were applied on a Bio-Gel A 150 M gel filtration column and fractions were analyzed for cytochrome *c* oxidase activity, R. C. index, phospholipid and size (Fig. 5). A significant heterogeneity existed in the size of the liposomes and the diameter varied over 50–300 nm (see below). All cytochrome *c* oxidase activity was associated with phospholipid. Depending on the diameter of the liposomes the protein/lipid ratio varied. The interesting phenomenon was observed that the R. C. index increased drastically with a decrease in diameter of the liposomes. The proteoliposomes were also subjected to a density gradient (Fig. 6). Again a close correlation existed between the cytochrome *c* oxidase activity and the phospholipid content. The variation in the density also reflected heterogeneity of the liposome preparation. Proteoliposomes with no cytochrome *c* oxidase activity could not be detected. The R. C. index increased with decreasing density of the liposomes.

Electron microscopy of proteoliposomes

Proteoliposomes containing *B. subtilis* cytochrome *c* oxidase (lipid/protein molar ratio 2200:1) made in the presence of calcium during dialysis showed a significant heterogeneity in diameter. This is shown in electron micrographs of negatively stained specimens of pooled fractions from the Bio-Gel A 150 M column. The diameter observed varied over 50–300 nm (Fig. 7).

The transmembrane pH gradient in cytochrome c *oxidase proteoliposomes and membrane vesicles*

Besides vectorial translocation of charge, proton translocation and intravesicular consumption of protons leading to water formation could also occur during the cytochrome *c* oxidase reaction. This would lead to the generation of a pH gradient across the membrane. The stimulation of the electrical potential by low concentrations of nigericin was already indicative for the presence of a pH gradient in these proteoliposomes. Direct evidence that in cytochrome *c* oxidase proteoliposomes a pH gradient was formed was obtained from distribution measurements of the weak acid [U-^{14}C]acetate using flow dialysis (Fig. 8). Upon addition of the electron donor ascorbate/$Ph(NMe_2)_2$/cytochrome *c*, a pH gradient of 46 mV was generated. Valinomycin, which specifically dissipates the electrical potential, increased the *Δ*pH to 54 mV. Similar results were obtained using pyranine fluorescence as internal pH indicator. The results are shown in Table 3. In proteoliposomes containing beef-heart or *B. subtilis* cytochrome *c* oxidase an internal alkalinization could be detected of 0.55 and 0.52 pH-units, respectively. The *Δ*pH increased upon the addition of low concentrations of valinomycin (10–20 nM) up to 0.68 and 0.63, respectively. The changes of the external pH, as recorded with a pH electrode, in the presence of ascorbate/$Ph(NMe_2)_2$/cytochrome *c* were maximally 0.02. The intravesicular pH changes in *B. subtilis* cytoplasmic membrane vesicles measured by pyranine

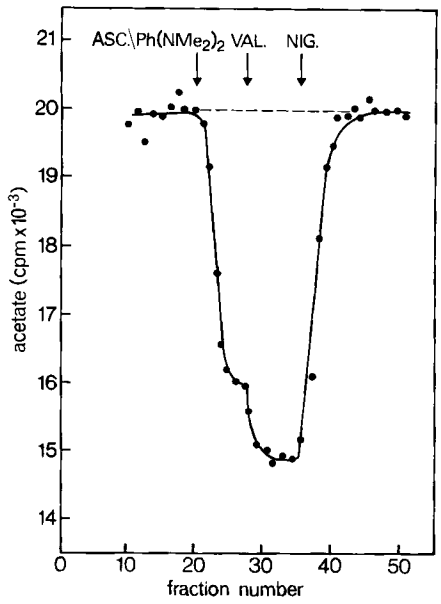

Fig. 8. *Determination of ΔpH, generated by proteoliposomes containing beef-heart cytochrome* c *oxidase by measuring the uptake of acetate using flow dialysis.* Flow dialysis was performed as described in Methods using proteoliposomes (6 mg asolectine) in an oxygen-saturated medium containing 270 μM [U-^{14}C]acetate (spec. act 2.0 TBq/mol). Ascorbate (Asc., 20 mM), $Ph(NMe_2)_2$ (200 μM) and cytochrome *c* (20 μM) was used as electron donor. Valinomycin (2 μM) and nigericin (1 μM) were added at the time indicated

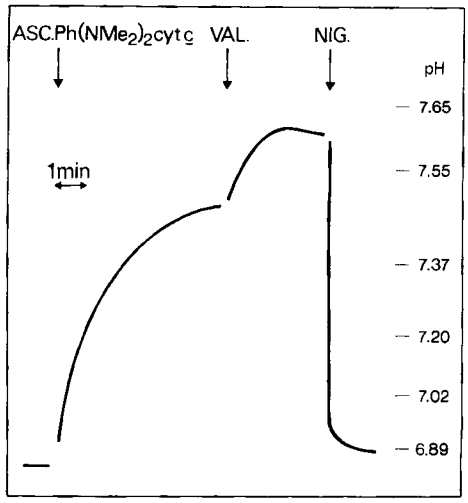

Fig. 9. *Measurement of pyranine fluorescence in* B. subtilis *cytoplasmic membranes with cytochrome* c *oxidase as Δp-generating system. B. subtilis* membranes (0.9 mg protein) with entrapped pyranine (200 μM) were added to 1.5 ml oxygen-saturated 50 mM potassium phosphate buffer, pH 7.0, 5 mM $MgSO_4$. Ascorbate (ASC., 10 mM), $Ph(NMe_2)_2$ (200 μM) and cytochrome *c* (10 μM) were added as electron donor at the times indicated. Valinomycin (1 μM) and nigericin (0.5 μM) were added to dissipate *Δψ* and *Δ*pH, respectively. Calibration was performed by the addition of KOH to uncoupled membranes as described

Table 3. *Quantification of ΔpH (interior alkaline) in cytochrome-c-oxidase-containing proteoliposomes and* B. subtilis *membrane vesicles* Pyranine fluorescence measurements were performed as described (see Fig. 9). Ionophores: val. = valinomycin, nig. = nigericin

Electron donor	Ionophore	$-Z\,\Delta$pH in		
		proteoliposomes of		*B. subtilis* membrane vesicles
		B. subtilis	beef heart	
		mV		
Ascorbate (10 mM) + $Ph(NMe_2)_2$ (200 μM) + cytochrome *c* (10 μM)	–	−32	−34	−29
	+ val. (20 nM)	−39	−42	−46
	+ nig. (50 nM)	0	0	0

fluorescence (Fig. 9) upon addition of the electron donor ascorbate/$Ph(NMe_2)_2$/cytochrome *c* were in the same order as observed in proteoliposomes. In these preparations valinomycin concentrations as high as 0.5 μM were necessary to observe an interconversion of the electrical potential into a pH gradient.

DISCUSSION

In this paper we have shown that purified cytochrome *c* oxidases from beef-heart mitochondria and from the bacterium *B. subtilis* incorporated in artificial membranes can generate a proton motive force composed of an electrical gradient (inside negative) and a pH gradient (inside alkaline).

In this study the *Δψ* was quantified with a Ph_4P^+-sensitive electrode. Accurate estimates of the *Δψ* from the distribution of Ph_4P^+ requires correction for Ph_4P^+ binding but this aspect has been considered carefully only in a few studies [26, 27]. To avoid complications the *Δψ* was measured under conditions where binding of Ph_4P^+ was relatively low and linearly dependent on the free Ph_4P^+ concentration up to 200 μM.

A characteristic of the probe molecule is that it responds to any potential which exists across the membrane such as a transmembrane bulk *Δψ*, a Donnan or a surface potential. This phenomenon could have an important effect on Ph_4P^+ uptake in liposome preparations with a high curvature at low ionic strengths. Due to this high curvature at low ionic strengths most probably a Donnan or surface potential will exist caused by the charge dislocation of head groups of the phospholipids on the membrane surfaces. This would lead to an overestimation of probe binding and therefore to an underestimation of the calculated *Δψ*. These surface phenomena could be abolished by increasing the ionic strength or the average diameter of the liposomes. In addition an increase of the average diameter of the liposomes leads also to a decrease of the asymmetry of binding. This is reflected in decreased binding constants in larger liposome preparations (see Table 1), although a certain effect of calcium on this binding cannot be excluded.

An argument in favour of the correction model is given in Fig. 1. When precautions were taken to circumvent high probe

binding a very good correlation was observed between the theoretical and the calculated $\Delta\psi$. When under these circumstances no correlation for concentration-dependent probe binding was applied the calculated $\Delta\psi$ would even be higher than the theoretical possible $\Delta\psi$. Presumably even a better correlation between calculated and generated $\Delta\psi$ could be obtained in proteoliposomes due to decreased binding constants. Heterogeneity with respect to the cytochrome *c* oxidase content of the liposomes could also lead to significant errors in quantification of the gradient. However, density gradient and gel filtration analysis did not reveal a detectable fraction of oxidase-free liposomes.

B. subtilis cytochrome *c* oxidase has a relatively simple polypeptide structure in comparison with the eukaryotic enzyme. The functional characteristics in generation of a Δp were more or less equivalent. Although the turnover rates of both enzymes in reconstituted form vary significantly, the observed R. C. index were about equal. This phenomenon could indicate slip or a higher ion permeability of the proteoliposomes containing beef-heart cytochrome *c* oxidase than those containing *B. subtilis* oxidase. The R. C. index varied significantly with the diameter of the proteoliposomes: the smaller the liposome the higher the index. The same phenomenon was observed by Madden and coworkers [45]. Increase of the protein content per vesicle resulted in a rapid decline of the coupling ratio. They suggested that protein-protein interactions or inwardly oriented oxidase molecules generate defects in the membrane through which ions could pass.

Reduced cytochrome *c* (E_m = + 210 mV) and also reduced PMS (E_m + 60 mV) could be used in both enzyme preparations as an effective electron donor. Reduced PMS is very often used as an electron donor for bacterial electron transport chains. PMS is membrane-permeant in both reduced and oxidized form [44] but still a right-side-out oriented Δp (inside negative and alkaline) is generated in these proteoliposomes, despite a significant fraction of inside-out oriented cytochrome *c* oxidase molecules. A similar observation has been made with *Escherichia coli* cytochrome-*o*-containing liposomes, but no information is available about the orientation of the incorporated cytochrome *o* molecules [43]. It seems likely that the rate of membrane permeation of PMS is much slower than the oxidation rate of the reconstituted enzyme with its substrate binding site at the external surface of the membrane.

Direct measurements of the pH gradient with two independent techniques, flow dialysis and pyranine fluorescence, showed that the proteoliposomes generate a significant pH gradient across the artificial membrane. Only a slight discrepancy of the calculated gradients as determined with both techniques was observed. Pyranine fluorescence was used for quantification of the pH gradient in the proteoliposomes and membrane vesicles. Pyranine is a hydrophilic molecule that does not bind to anionic artificial membranes [28]. Since this probe does not leak out readily from liposomes, it is an excellent indicator for intraliposomal pH. A limited interconversion of the $\Delta\psi$ into a pH gradient was observed which could be explained by a relatively high buffer capacity for protons in comparison to the capacity for charged species in the proteoliposomes. The extrapolated maximal Δp (i.e. $\Delta\psi$ in the presence of low concentration of nigericin) varied between − 110 mV and − 140 mV (Fig. 3). The efficiency of energy transduction can be calculated from these data. The free energy (ΔG) in the enzymic reaction comprises about 600 mV at 50% reduction of cytochrome *c* (E_m = 210 mV) to oxygen (E_m = + 800 mV). When the thermodynamic stoichiometry equals one proton transferred per electron the efficiency of energy transduction lies in between 18% and 23%. If cytochrome *c* oxidase could function as a proton pump, the H^+/e stoichiometry would be 2 and the efficiency would increase by a factor 2. Cytochrome *c* oxidase would then be a very efficient Δp-generating system.

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